

## Symposium on Bacterial and Viral Genetics

A symposium on bacterial and viral genetics, organized by the Department of Microbiology of the Australian National University, was held in the new Lecture Theatre of the John Curtin School of Medical Research on Monday August 19 1957. It was supported in part by a grant from the Australian Academy of Science, and was attended by members of the Genetics Society of Australia and by microbiologists. There were visitors from all States as well as from England and U.S.A.

*Genetics of Vaccinia Virus*: paper by Professor Frank Fenner.

*Exchange of Character Determinants between Strains of Plant Viruses*: paper by Dr. R. J. Best.

### Introduction

JOSHUA LEDERBERG\*

In genetic analysis, recombinational phenomena play a central role. In order to explore the genetic material, we must expose it to experimentally defined reagents. Ultimately, we shall learn more from chemical reagents, but at present the most fruitful experiments in

CATEGORY.	UNIT OF EXCHANGE.	EXAMPLES.
1. Heterokaryosis	Intact nucleus	<i>Streptomyces griseus</i> .
2. Sexuality	Fusion nucleus (entire genome or major part)	<i>Escherichia coli</i> , <i>Pseudomonas aeruginosa</i> , <i>Streptomyces coelicolor</i> .
3. Transduction	Genotypic fragment	
(a) DNA mediated		pneumococcus; <i>Hemophilus</i> .
(b) Phage mediated		
i. Generalized		Salmonella; <i>E. coli</i> : phage Pl.
ii. Prophage-linked		<i>E. coli</i> (lambda-Gal).
iii. Prophage	(= lysogeny or lysogenic conversion)	

A summary of the papers delivered follows. In the evening there was a general discussion which was introduced by Dr. Joklik's description of the application of autoradiography to problems of DNA replication ('astrobiology'), and was concerned largely with speculation on the chemical basis of heredity.

*Introduction*: paper by Professor Lederberg.

*Genetic Recombination in Pseudomonas aeruginosa*: paper by Dr. Holloway.

*Lysogenicity and Transduction in Escherichia coli K12*: paper by Dr. Esther M. Lederberg.

*Genetics of Influenza Virus*: paper by Sir Macfarlane Burnet.

genetics involve the reactions of genes with one another, that is, recombination experiments.

Enough is now known of the genetic systems of different bacteria to permit a tentative classification in terms of the quality of the unit subject to exchange:

More can be said about each of these categories. In the *E. coli* sexual system, for example, Jacob has forwarded evidence that the act of mating may be interrupted by blending the bacterial suspension, so that fertilization is incomplete. He and his colleagues have reported the most skilful experiments which correlate the number of

\* Department of Medical Genetics, University of Wisconsin. (Fulbright Visiting Professor of Bacteriology, University of Melbourne.)

genes from the male parent which appear in a sexual progeny, and the amount of  $^{32}\text{P}$ -labelled DNA which it contains. Other evidence suggests that some matings, if left undisturbed, proceed to virtually complete fertilization, and there is still much to be learned in the quantitative evaluation of the various steps: the extent of initial transfer, the efficiency and uniformity of pairing, and especially the mechanism of 'incorporation' or crossing-over.

Dr. Holloway will bring us up to date on his studies of the *Pseudomonas* system, and Dr. E. M. Lederberg will elaborate on the prophage-linked transduction of Gal markers in *E. coli*.

The pioneering work on recombination in animal and plant viruses is a distinctively Australian contribution, and I am looking forward to the papers of Sir MacFarlane Burnet, Professor Fenner and Dr. Best for my own information on these fascinating developments.

#### Genetic Recombination in *Pseudomonas aeruginosa*

B. W. HOLLOWAY\*

In view of the unusual nature of the genetic processes that occur in bacteria it is desirable that a range of bacteria be studied in order to determine the generality of the mechanisms. The techniques developed by Lederberg for *E. coli* are applicable to certain other bacteria and have proved successful with *Pseudomonas*

TABLE I

The Formation of Prototrophs in Crosses between Auxotrophs in Four Strains of *Pseudomonas aeruginosa*

Strain	I	L	3	29
I .. ..	—	+	+	+
L .. ..		±	—	±
3 .. ..			—	—
29 .. ..				—

+ = recombination.

± = recombination at low frequency.

— = no recombination.

*aeruginosa*, a Gram-negative bacillus characterized by the formation of blue and green pigments.

*P. aeruginosa* will grow on a medium consisting of glucose and mineral salts. Auxotrophic mutants for a range of amino acids, purines and pyrimidines were isolated using manganous chloride as the mutagen. In certain cases crosses between different auxotrophic mutants resulted in the formation of recombinant prototrophs. The interfertility of the four strains used is shown in Table I.

Different mutant combinations of any two fertile parents gave different recombination

frequencies of prototrophs (for example see Table II).

Segregation of various non-selective markers has also been shown and the frequent formation of novel combinations of non-selective markers provides conclusive evidence of some genetic mechanism.

For example, in the cross I ( $\text{T}_1\text{-IV}_1\text{-S}^+\text{C}^+$ )—a tryptophane requiring, streptomycin sensitive, chloramphenicol resistant mutant of strain I— $\times$  L ( $\text{IV}_1\text{-T}_1\text{-S}^+\text{C}^+$ )—an isoleucine plus valine requiring, streptomycin resistant, chloramphenicol sensitive mutant of strain L—the formation of recombinants which are genotypically  $\text{T}^+ \text{IV}^+ \text{S}^+\text{C}^+$  can only be explained by genetic reassortment of the marker genes.

TABLE II  
Recombination Frequencies of I (LIL<sup>-</sup>) with various Auxotrophs of Strain L

Cross	Recombinants/10 <sup>8</sup> total parental cells
I (LIL <sup>-</sup> ) $\times$ L ( $\text{T}_1$ ) ..	20
$\times$ L (Ad <sup>-</sup> ) ..	55
$\times$ L (Thr <sup>-</sup> ) ..	1
$\times$ L (IV <sup>-</sup> ) ..	200

I (LIL<sup>-</sup>) = auxotroph of strain I requiring leucine.

T = tryptophane.

Ad = adenine

Thr = threonine.

IV = isoleucine and valine.

There is considerable evidence that the mechanism of recombination is very similar to that occurring in *E. coli*. In both organisms actual contact of the parental bacteria is necessary for genetic recombination to take place. There is a correlation between the F fertility system in *E. coli* and the interfertility of the various strains shown in Table 1. For the combination I  $\times$  L, with which much work has been done, equation of strain I as the F<sup>-</sup> and strain L as the F<sup>+</sup> gives a situation identical with that of *E. coli*. In both organisms there is an unequal contribution of the parents to the recombinant, the F<sup>-</sup> parent contributing more than the F<sup>+</sup>. Thus the recombinants tend to resemble more the F<sup>-</sup> parent. Strain I and strain L differ in their pigment forming ability, colonial morphology and in their reactions to a group of bacteriophages. 99 per cent. of recombinants resemble the strain I parent in these characteristics. Irradiation with ultraviolet light of the F<sup>+</sup> strain of both organisms increases recombination frequency, whereas no such effect occurs with the F<sup>-</sup> strain. In *E. coli* the F factor is infectious being easily transferred from a F<sup>+</sup> to a F<sup>-</sup> strain. Infectious transfer of this nature has been shown for *Pseudomonas* in preliminary experiments at low frequency. The paucity of suitable markers in *P. aeruginosa* has limited the linkage

\* Department of Bacteriology, University of Melbourne.

studies. It has been possible to divide the available markers into two groups, those which are inherited freely from the  $F^+$  parent by the  $F^-$  parent and those which fail to be inherited in this fashion. On the assumption of linearity it has been possible to establish a provisional chromosome map of the former group. Little is known yet of the actual processes of conjugation and genetic transfer in *Pseudomonas*.

Current work with *P. aeruginosa* is aimed at learning more of the actual mechanism of genetic transfer and also the manner of the inheritance of prophages from a multi-lysogenic strain which has been derived from strain L.

#### Lysogenicity and Transduction in *Escherichia coli* K-12

ESTHER M. LEDERBERG\*

The relationship termed lysogenicity is characteristic of bacteria which harbor viruses or phages in a latent form. Such temperate phages can undergo either a lytic cycle whereby the host is destroyed immediately or can allow the production of a viable bacterial clone which liberates the phage sporadically over an indefinite number of generations. This process occurs stochastically in an untreated population, and may be greatly accelerated by mutagenic agents.

The starting point of genetic analysis of lysogenicity is its transmission in the non-virulent stage, *prophage*, during vegetative reproduction as part of the hereditary constitution of each cell. The accidental discovery of lysogenicity in *E. coli* K-12 in which sexual recombination had been demonstrated furnished a unique opportunity to study its genetic basis.

The demonstration of lambda, the temperate phage carried by strain K-12 became possible when a rare mutation leading to loss of prophage had occurred. This was associated with loss of immunity and thus susceptibility to lysis by lambda. The main phenotypes include  $Lp^+$  (lysogenic),  $Lp^s$  (sensitive), and  $Lp^i$  (immune). The latter carried a defective prophage, conferring immunity or resistance to lysis, but no phage active against  $Lp^s$  is produced.

Intercrosses in every combination of these types resulted in a strict segregation of the ensuing progeny into just the parental classes, suggesting the existence of an  $Lp$  locus for the maintenance of lambda prophage at a specific site on the chromosome. The chromosomal hypothesis was strengthened by linkage tests in crosses involving a number of genetic markers: a close linkage of  $Lp$  to a cluster of loci controlling galactose fermentation ( $Gal$ ) was found. The linkage was verified by the linked segregation of  $Lp$  and  $Gal$  from heterozygous diploids of several types. Segregation patterns were also followed in homozygous diploids:  $Gal-Lp^s/Gal-Lp^s$  which were first infected with lambda and then allowed to

revert to  $Gal^+$ . The infected diploids were lysogenic, showing that  $Lp^+$  was dominant to  $Lp^s$ , but both were recovered as haploid segregants. Thus the fixation of the prophage had occurred on one of the two chromosomes.  $Gal^+$  reversions were then selected under the appropriate conditions. The diploid then segregated  $Gal^+/Gal^-$  as well as  $Lp^+/Lp^s$ . The reversion had occurred either on the  $Lp^+$ —or  $Lp^s$ —marked chromosome (coupling phase or cisdiploid vs repulsion phase or trans diploid). A few crossover recombinants were also identified.

On one hypothesis lysogenicity is determined by the cytoplasm. It would be expected that the diploid cell would provide the most favorable opportunity for the intermingling of the cytoplasm and would be detected by the uniformity of the haploid segregants with regard to their lysogenic character. However, the segregation of  $Lp$  and  $Gal$  as an intact part of the linkage group confirms the alternative hypothesis of integration of lambda prophage into a definite site on the chromosome.

Another link between phage and bacterial genes is the process of genetic transduction which is defined as the transfer of a fragment of the genotype in contrast to sexual recombination which involves an essentially intact nucleus. The transduction mediated by lambda differs from the generalized transduction described for *Salmonella* in several respects: (1) effective transducing lambda arises only by induction of  $Lp^+$  bacteria, not by lytic growth on  $Lp^s$ ; (2) the transfer is confined to the  $Gal$  loci linked to  $Lp$ ; (3) the transduction clone is unstable: a partial diploid or *heterogenetic* stage continues to segregate  $Gal^+/Gal^-$  but no other markers; (4) the recipient may be lysogenic, immune, or sensitive, providing that lambda is adsorbed; (5) lysates produced from heterogenotes are HFT, i.e., every particle is potentially capable of initiating a transductional event, in contrast to LFT lysates ('low frequency of transduction') from haploid donors.

The donor bacterium can be marked by a genetically different  $Gal^-$  ( $a^-b^-$ ) than the recipient ( $a^-b^+$ ). A  $Gal^+$  heterogenote ( $a^-b^-/ex\ a^-b^-$ ) is formed and segregates in order of decreasing frequency, recipient or endogenetic type ( $a^-b^-$ ), donor or exogenetic type ( $a^-b^-$ ) and double  $Gal^-$  crossover type ( $a^-b^-$ ). Rearrangement in a heterogenetic cell sometimes results in homogenotes carrying the same  $Gal$  allele on the exogenote and endogenote. Specific  $Gal^-$  HFT lysates may be produced from these and exploited in homology or allelism tests. Each  $Gal^-$  culture is transformed to  $Gal^+$  by the HFT lambda from  $Gal^+$  or any nonallelic  $Gal^-$  donor. The results were fully consistent with those from standard crossing experiments.

The ten distinctive loci controlling the fermentation of galactose so far identified had been isolated as unique mutational events. Certain combinations of  $Gal^-$  formed phenotypically mutant heterogenotes ( $a^-b^-/a^-b^-$ ).

\* University of Wisconsin.

Rearranged heterogenotes of the constitution:  $a^+b^-/a^-b^+$  were phenotypically  $\text{Gal}^+$ , indicating a cis-trans position effect. Combinations of a mutant from one position-effect group or cistron with one from the other are  $\text{Gal}^+$ ; within the cistron, the heterogenotes are  $\text{Gal}^-$ . Two position-effect groups have been identified genetically and their enzymatic defect determined: Gal 2, 8, 10 mutants are defective in galactokinase; Gal 1, 4, 6, 7, 11, in UDP-hexose-transferase. The latter carry the same defect as galactosemia in man. The enzymology of a third group, (Gal 3, 9), which give position-effects with either of the other groups is not yet known.

A close correlation of transduction and lysogenization was observed. From  $\text{Lp}^s$  recipients either pure  $\text{Lp}^+$  or  $\text{Lp}^{+s}$  heterogenotes are found. The latter are nonlysogenic, immune to lambda, but every segregant is  $\text{Lp}^s$ . Several hypotheses advanced to account for them are under critical test. Transductions involving the addition of prophages related to lambda as other markers for the recipient show the following significant features: (1) heterogenotes segregate lysogenic/sensitive for either or both prophages along with two Gal loci; (2) linkage of Gal type and lambda marker; (3) continued segregation of one or both prophages in segregants already pure for Gal. Super-infection of a lysogenic with related phages resulted in either unstable double lysogenics or prophage substitution.

In the light of these findings lysogenization may be regarded as a more restricted form of transduction: the phage behaves as the vector as well as the passenger in the transduction of lysogenicity.

### Genetics of Influenza Virus

F. M. BURNET\*

For the past seven years extensive studies on the genetic interaction of strains of influenza virus have been carried out at the Hall Institute. As a preliminary to any work on recombination it is necessary to establish the conditions needed to isolate and maintain pure clones of virus. The use of limiting infective dilutions for this purpose was first worked out in relation to the changes which take place when an influenza virus is adapted to growth in chick embryo (the O-D phenomenon). The general principles involved in the appearance of mutants and the selective survival of mutant clones have been studied in a number of papers, notably the investigations of Edney (1957) on the progressive replacement of an original strain by serum-resistant variants in the presence of homologous immune serum.

For any type of genetic work it is necessary to have clearly defined differences between the forms used for hybridization and in general it is helpful to have available a range of strains showing many 'marker' characters. In viruses it is particularly valuable to have marker difference which can be detected by simple

*in vitro* tests. Many such characters can be defined for influenza virus strains by using the haemagglutinating activity of the virus as an index. For all practical purposes the most important character of a virus is its virulence for its natural and laboratory hosts and another series of marker differences will be based on virulence for various host tissues or cells.

In our work the following markers have been employed:

- I. Based on haemagglutination
  - Aa Serological character
  - Bb Heat stability of haemagglutinin
  - Cc Inhibition of heated haemagglutinin by ovomucin, etc.
  - Dd Inhibition of heated haemagglutinin by sheep salivary mucin.
- II. Virulence characters
  - Ee Capacity to produce haemorrhagic lesions in chick embryo.
  - Ff Extent of lung lesions after intranasal inoculation of mice.
  - Gg Pathogenicity on intracerebral inoculation in mice.
- III. Morphology
  - Hh Proportion of filaments in standard infective fluid.

When conditions are arranged so that susceptible cells can be simultaneously infected with two different influenza virus strains, the following types of genetic interaction may be recognized:

1. Transfer of neurotropism from NWS to other serological types of influenza A (Burnet and Lind, 1949).
2. Reciprocal recombination within the MEL/WSE system with definition of 2 'linkage groups'.
3. Redistribution of virulence in some systems, not in others.
4. Phenomenon of double neutralization—phenotypic mixture.
5. 'Resurrection' of virus killed by heat or UV by appropriate recombination.
6. Phenomenon of heterozygosis.

Recent work has been concerned largely with a phenomenon which seems to involve the action of a suppressor gene. A strain of influenza virus, WSE, highly virulent for mice, gave rise to a mutant completely lacking in virulence. This avirulent strain, aWS—, was genetically stable and could be passed without change in any of its properties indefinitely at limit dilution. If, however, passage from one embryo to another is made by inoculation of the allantoic cavity with relatively concentrated infective fluid, mouse virulent mutants appeared after a few generations. Analysis of the process indicates that the reversion to virulence takes place by a series of steps. It is possible to obtain revertants to full mouse virulence without the necessity of passage

\* Walter and Eliza Hall Institute, Melbourne.

through mice. It seems evident therefore, that there has been no true genetic loss of virulence in the change from WS— to aWS— and some form of masking or suppressor effect must be postulated.

The problem of stabilizing virulence or lack of virulence in virus strains is often a matter of great practical importance. The existence of strains of the aWS— type showing masked virulence indicates one of the major difficulties in being sure that any attenuated variant has been 'fixed' in the avirulent form.

### Genetic Studies with Vaccinia Virus

FRANK FENNER\*

Vaccinia virus is one of the larger animal viruses, and differs from influenza virus in that its genetic material is DNA and not RNA. Thanks to Edward Jenner it has been used by man for a longer period than any other virus, and strains with the most varied passage histories are maintained in different laboratories all over the world. Twenty four such strains have been intensively examined in my department over the last two years, and considerable differences have been found in several biological properties of different strains of vaccinia, cowpox, and rabbitpox viruses—all of which are very closely related immunologically.

Six groups of characters were studied, two *in vitro* characters—production of haemagglutinin and sensitivity to inactivation by heat; and four *in vivo* characters—pock production on the chorioallantoic membrane, growth in various types of tissue culture, the type of lesion produced after intradermal inoculation of rabbits, and virulence for mice after intracerebral inoculation. With one or two exceptions all strains examined were distinguishable from each other. Two pairs of strains with a number of contrasting characters were selected for experiments on recombination. The pairs, and the contrasting characters are shown in Table I.

The most extensive experiments have been carried out with rabbitpox and dermal vaccinia.

\* Department of Microbiology, John Curtin School of Medical Research, Australian National University.

The first experiments involved the inoculation of the chorioallantoic membrane with large doses of the two parent strains and assay of the new virus produced twelve hours later. Five recombinant clones were found among the 95 clones tested.

By inoculating eggs with mixtures calculated to produce about 50 pocks of each type it was possible to obtain a few membranes on which two adjacent pocks of different type overlapped. From the overlapping areas clones of virus were obtained by single pock isolation. About

TABLE II

Examples of the stable recombinants obtained in experiments with rabbitpox and dermal vaccinia viruses. The abbreviations are shown in Table I. Underlining indicates the features in which the strain in question differs from the parent dermal vaccinia strain

Strain	CAM	MV	RS	HA	HR
rabbitpox parent	R	h	PC	0	h
dermal vaccinia parent	W	l	N	h	l
R1-1	W	l	N	0	l
R1-3	W	<u>int.*</u>	<u>int.</u>	<u>h</u>	l
R1-4	W	<u>l</u>	<u>N</u>	h	<u>h</u>
R2-3	W	<u>int.</u>	<u>int.</u>	h	<u>h</u>

\* int. = intermediate between the behaviour of the two parent strains.

30 per cent. of the clones obtained showed a grouping of characters differing from that of each of the parents. Some clones were stable on repeated passage and others varied somewhat. Sometimes the *in vivo* characters differed from those of both parent strains, but no 'intermediates' were obtained in heat resistance or haemagglutinin production. Recombination was also demonstrated between neurovaccinia and the white variant of cowpox.

Other lines of investigation were concerned with mutation in the poxvirus group—the 'white variants' of cowpox and of some of the neurovaccinia strains providing material for the Luria-Delbrück fluctuation test. The process of adaptation of dermal vaccinia strains to rapid growth in mouse brain was also being

TABLE I  
The biological characters of the parent strains of the pairs used for recombination experiments

Strain	Chorioallantoic Membrane		Intradermal Inoculation of Rabbits (RS)	Intracerebral Inoculation of Mice (MV)	Haemagglutinin Production (HA)	Heat Resistance (HR)
	Pock Appearance (CAM)	Virus Content of Pock (VC)				
rabbitpox	pale haemorrhagic (R)	high (h)	indurated lesion with purple centre (PC)	highly virulent (h)	nil (0)	high (h)
dermal vaccinia	opaque white (W)	low (l)	red nodule (N)	non-virulent (l)	high (h)	low (l)
neurovaccinia	pale haemorrhagic (R)	high (h)	indurated lesion with purple centre (PC)	highly virulent (h)	high (h)	high (h)
cowpox-white variant	opaque white (W)	low (l)	red nodule (N)	non-virulent (l)	very low (l)	high (h)

examined. In one case there appeared to be a single step change from low to high mouse virulence, and this was correlated with a pronounced change in pock appearance. In other cases there appeared to be a gradual increase in virulence not clearly correlated with a change in pock appearance.

#### Exchange of Character Determinants between Strains of Plant Viruses

R. J. BEST\*

The exchange of character determinants between strains of a virus was first demonstrated for bacterial viruses, and later for influenza virus. Some bacterial and animal viruses have now been studied sufficiently for genetic terms and techniques to be used with assurance and profit. So far satisfactory evidence that a similar phenomenon occurs in plants has been obtained for only one virus—the virus of tomato spotted wilt (TSW). The complicated nature of multicellular hosts and the fact that a plant virus lesion becomes apparent only after several virus generations, means that the general use of genetic terms at this stage is unjustified and perhaps presumptuous. However, the evidence seems to be clear that strains of TSW virus exchange determinants when they multiply together in plants, and that the new strains which we have isolated from mixedly infected plants combine some of the characters of their 'parents'.

TSW. virus is spherical, with a diameter of about 85 m $\mu$ , has a thermal death point of 46°C, and has an *in vitro* life of less than 12 hours in the sap expressed from infected plants. However, by using reducing agents to keep the redox potential of solutions below the critical value for the virus and by working in buffered solutions at 2°C, we can keep the virus infective for a reasonable time. The virus is readily transmitted by mechanical inoculation. Chemical analysis and ultra-violet absorption correspond to a nucleoprotein of intermediate P content. Although chemical and serological tests are not as yet available to us as markers for different strains, the biological properties

as expressed in tissue specificity and symptomatology are so definite and unequivocal that very useful information can be obtained through them alone. The diagnostic symptoms by which we recognise the strains are a type of biochemical indicator, and it is assumed that each diagnostic character is related to a determinant which is part of the virus particle.

Over the years a number of naturally occurring strains have been isolated and characterized. These breed true and have been maintained by serial transfer at regular intervals (about fortnightly) over a period of years. Only those characters which are invariably associated with a strain are used in this work.

When two of these strains (A and E) were inoculated simultaneously onto the same plants, a number of new strains (as well as the originally introduced A and E) were recovered from the mixedly infected plants. The characters of two of these new strains are tabulated along with those of strains A and E in Table I.

Both new strains (isolated by limit dilution technique) differ from the originals but combine some of the characters of each. Strain 'etch' shows a character (Etch) not shown by either of the parents. Both new strains have been isolated from a number of different plants in one experiment and again in a similar experiment repeated some months later. The strain New 'B' has been isolated not only from mixedly infected tomato plants, but also from the systemically invaded tops of *N. glutinosa* plants, a host which only one of the parents (E) invades systemically.

The frequency with which the new strains (especially New 'B') occur on the mixedly infected plants in any one experiment and the fact that they appear whenever the experiment has been repeated makes it unlikely that they are mutants.

Other specially designed tests provided further evidence against the mutant possibility.

It is considered that the interpretation which best fits all the facts is that the new strains arose as a result of an exchange of determinants between the originally introduced strains.

TABLE I

Illustrating the transfer of character determinants between strains A and E of the virus of tomato spotted wilt; symbols represent diagnostic characters (on three hosts) of strains A and E and of new strains isolated from plants mixedly infected with strains A and E

Strain	<i>Lycopersicon esculentum</i>					<i>Nicotiana glutinosa</i>					<i>Nicotiana tabacum</i>		
	Primary NIP	Sy	Systemic Na	NIP	E	Primary NI	Yb	Sy	Systemic C	NIP	Primary NI	R	Systemic Sy
A .. .. .	+	+	+	+	—	+	—	—	0	0	+	—	—
E .. .. .	—	—	—	—	—	—	+	+	+	—	+	(+)	+
New B .. .. .	—	+	—	+	—	+	—	+	—	+	+	+	+
Etch .. .. .	—	+	—	—	+	+	—	+	—	+	+	(+)	+

NI = leaf neurosis.  
Na = apical neurosis.  
P = pigmentation.  
E = etch.

Sy = systemic invasion.  
Yb = yellow blotch.  
C = chlorosis.  
(R) = ringspot at high temperatures.